A natural variability in the proline-rich motif of Nef modulates HIV-1 replication in primary T cells

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In the infected host, the Nef protein of HIV/SIV is required for high viral loads and thus disease progression [1–3]. Recent evidence indicates that Nef enhances replication in the T cell compartment after the virus is transmitted from dendritic cells (DC) [4]. The underlying mechanism, however, is not clear. Here, we report that a natural variability in the proline-rich motif (R71T) profoundly modulated Nef-stimulated viral replication in primary T cells of immature dendritic cell/T cell cocultures. Whereas both Nef variants (R/T-Nef) downregulated CD4, only the isoform supporting viral replication (R-Nef) efficiently interacted with signaling molecules of the T cell receptor (TCR) environment and stimulated cellular activation. Structural analysis suggested that the R to T conversion induces conformational changes, altering the flexibility of the loop containing the PxxP motif and hence its ability to bind cellular partners. Our report suggests that functionally and conformationally distinct Nef isoforms modulate HIV replication on the interaction level with the TCR-signaling environment once the virus enters the T cell compartment.

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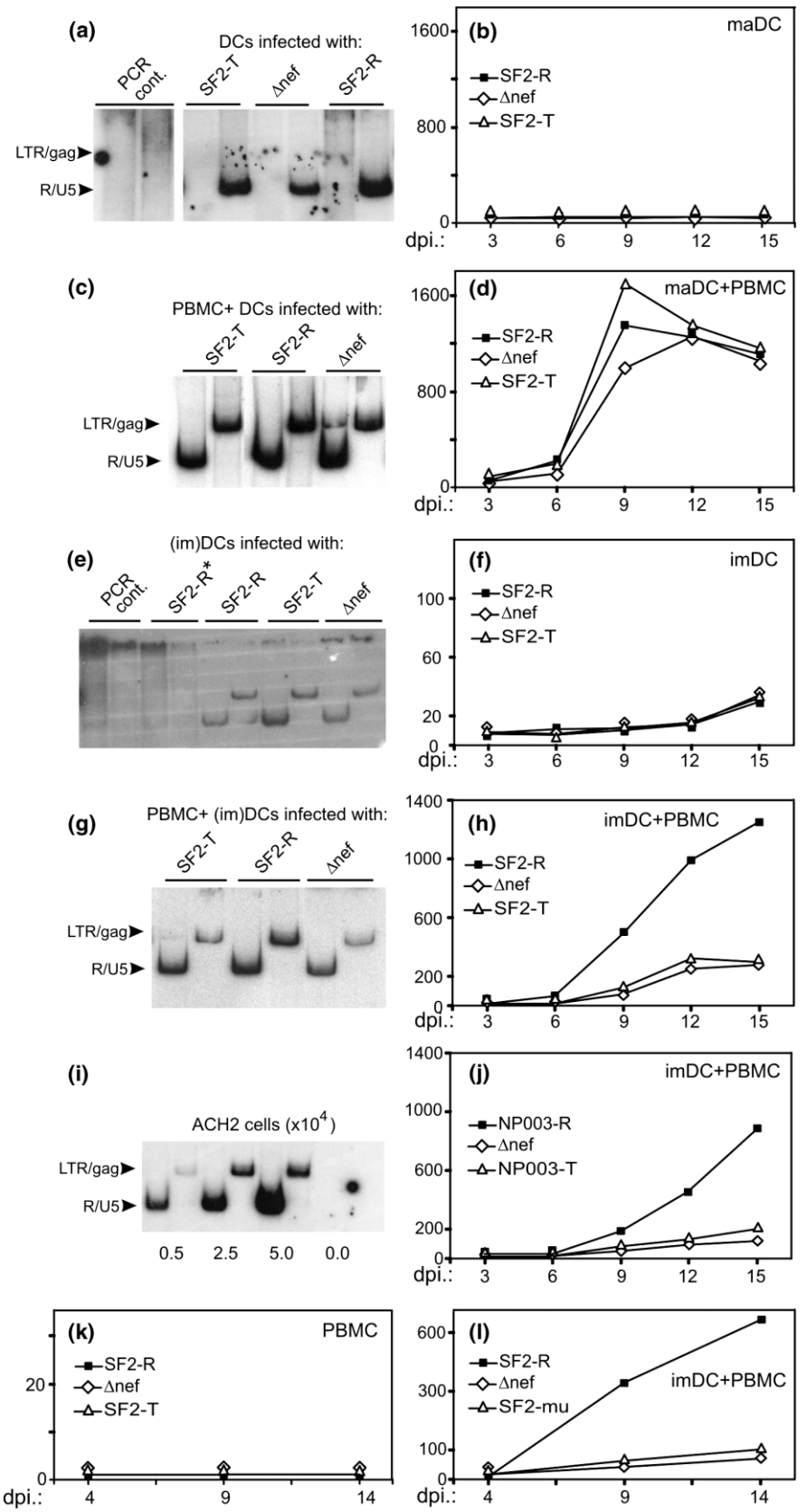
Results and discussion

Some *nef* alleles like IIIB and BH8 fail to support viral replication in primary cells in vitro [5]. In some cases, such as with NP003, this correlates with the lack of disease progression [6]. Like the closely related NL4-3, these *nef* alleles contain a natural variability that is only present in ~5% of all Nef proteins, namely a T (T^{71}) instead of an R in the otherwise conserved proline-rich domain (P^{69}xxPxxPxxP^{78}). Upon reversion of T^{71} to R^{71} in the primary allele NP003, replication was restored completely [6]. To confirm its importance, R^{71} was changed to T^{71} in the lab-allele SF2 and cloned into the NL4-3 backbone for analysis of replication kinetics. As with NP003, we found that the T variant of SF2 (SF2-T) was unable to replicate in HS-transformed primary T cells (see Figure S1 in the Supplementary material available with this article online).

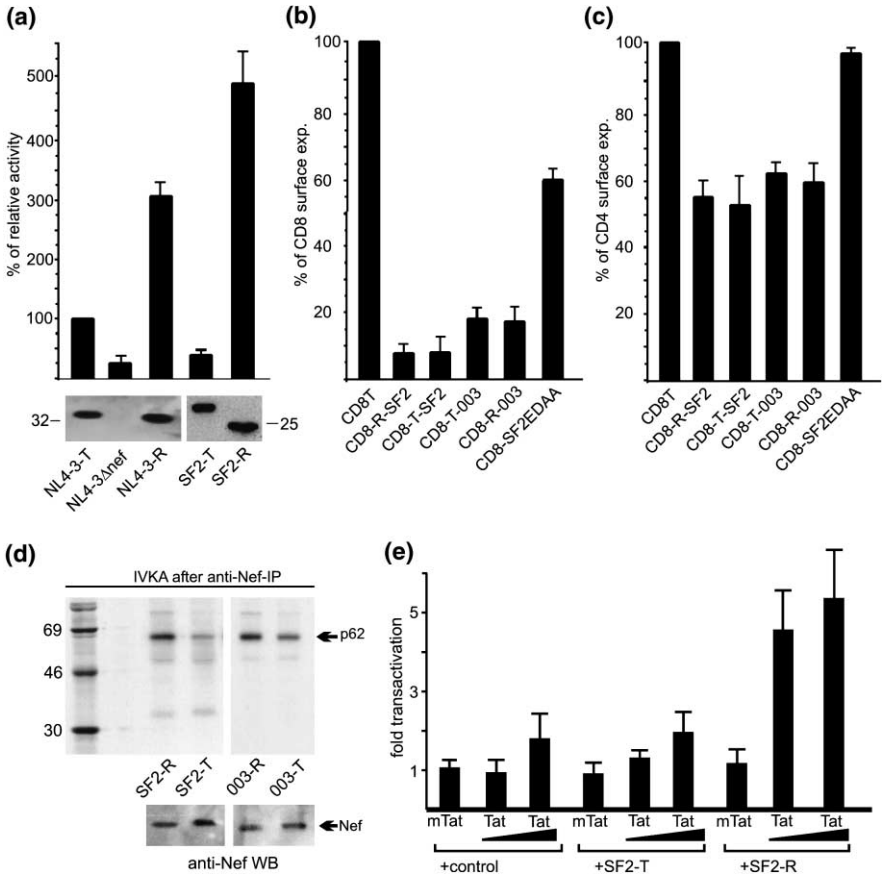
We initially sought to confirm these results in resting PBMCs alone; however, high infectious doses were required to induce virus replication, and the results were highly donor dependent and ranged from no effect of Nef to a requirement for R71 in Nef (data not shown). Therefore, we decided to use the physiologically more relevant autologous immature (im)-DC-T cell coculture system in which SIV replication has a strong requirement for functional Nef [4]. For this purpose, gp120 of NL4-3 was changed from T cell tropic to macrophage tropic (*env* from HIV Bal) in all viral constructs described above. Infection of DC and virus replication was monitored by PCR and reverse transcription assay [7, 8]. As anticipated [8, 9], mature (ma)-DC alone were unable to replicate HIV, and the PCR amplification yielded only LTR (R/U5 region) transcripts, indicating incomplete reverse transcription (Figure 1a,b). Likewise, infection of nonactivated PBMC alone with these low doses of virus input did not yield any viral replication (Figure 1k). However, viral replication was induced after adding nonactivated autologous PBMC to the infected ma-DC, and complete reverse transcripts were detected (LTR/*gag* region, Figure 1c,d). In agreement with previous observations, Nef had no effect in these cultures (Figure 1d) [4]. When im-DC alone were infected, low levels of HIV replication were measured with all constructs, consistent with the detection of early and complete reverse transcripts (Figure 1e,f). Adding PBMC to these cultures stimulated replication of the proviral clone bearing the wild-type SF2 allele (R-Nef). Conversely, viruses encoding T-Nef or lacking Nef failed

Figure 1



Requirement of R71 in Nef for optimal HIV replication in cultures of im-DC and T cells. Representatives of at least three experiments are shown, using cells (PBMC and DC) from the same donors for a given experiment. **(a)** ma-DC (5×10^4) were infected with recombinant viruses deleted in the *nef* gene (Δ*nef*) or coding for SF2-T- and SF2-R-Nef proteins, respectively. After 15 days of culture, only incomplete reverse transcripts (R/U5) could be amplified by PCR (right panel); but not in noninfected cells (PCR cont., left panel). **(b)** RT activity in culture supernatants of the experiment described in (a). **(c)** ma-DC were infected as in (a). One day after infection, 2×10^5 nonstimulated autologous PBMC were added. After 15 days of culture, both complete (LTR/gag) and incomplete reverse transcripts (R/U5) were detected by PCR. **(d)** RT activity in supernatants of the virus culture described in (c). **(e)** PCR analysis on day 15 after infection of im-DC (5×10^4) with the viruses described in (a). The original, T cell tropic, NL4-3 SF2-R virus was used (SF2-R*) as an additional control. **(f)** RT activity in culture supernatants described in (e). **(g)** PCR analysis on day 15 of immature im-DC infected as in (e) and cocultured with PBMC as in (c). **(h)** RT activity in culture supernatants described in (g). **(i)** To determine the sensitivity of the PCR reaction, reverse transcripts were amplified using DNA from increasing numbers of ACH2 cells, which contain one copy of provirus per cell. **(j)** RT activity in supernatants of an im-DC-PBMC coculture after infection of the DC with NL4-3(Bal *env*)virus coding for the R- and T-Nef pair of the NP003 primary *nef* allele. **(k)** Infection of 2×10^5 nonactivated PBMC by the above-described viruses did not yield measurable viral replication. **(l)** RT activity in an im-DC-PBMC coculture after infection of the DC with a recombinant virus coding for a SF2 *nef* allele with a mutation in the PxxP region (SF2-mu).

Figure 2



A comparison of the R and T isoforms in enhancement of virion infectivity, CD4 downregulation, and cellular stimulation by Nef. **(a)** Relative infectivities of the indicated viruses produced from 293T cells were determined in a single round of replication assay on HeLa-CD4-LTR-β-Gal indicator cells (Sx22-1) (top panel). Expression levels of the various Nef proteins were verified by Western blot (bottom panel). **(b)** FACS analysis of the steady-state surface expression of CD8-Nef fusion proteins in 293T cells. **(c)** FACS analysis of the steady-state surface expression levels of CD4 in 293 cells stably expressing CD4 after transfection with the same CD8-Nef fusion proteins as in (b). Standard errors of the mean from at least three independent experiments are indicated by error bars. **(d)** In vitro kinase assay after immunoprecipitation of CD8-Nef from transiently transfected Cos-7 cells (upper panel). The arrow points to the phosphorylated p62 PAK protein. The same nitrocellulose filter was probed with an anti-Nef antibody to verify the presence of comparable amounts of Nef (arrow) in these immunoprecipitates (bottom panel). **(e)** The transactivation activity of Tat or mutant Tat C30G (mTat) on an HIV-LTR-CAT reporter construct was analyzed in CV-1 cells transiently expressing increasing amounts of T- or R-Nef. Results are expressed as fold transactivation over the activity of mTat, and standard errors of the mean from three independent experiments are indicated by error bars.

to increase replication above a background level (Figure 1h). Importantly, a virus encoding SF2-R with mutations in the PxxP motif behaved like SF2-T or Δ*nef* viruses (SF2-mu, Figure 1l). In line with the replication data, PCR amplification of the LTR/*gag* region was increased 2- to 3-fold over SF2-T and Nef-negative (Figure 1g). Similar results were obtained using the primary NP003 Nef R/T pair (Figure 1j). In summary, it appeared that a threonine at position 71 greatly reduced the ability of Nef to support viral replication in nonstimulated T cells.

To analyze the mechanism, we compared R- and T-Nef proteins for established activities in vitro such as enhancement of virion infectivity, CD4 downregulation, and cellular activation. When the relative infectivity of the respective recombinant viruses was assayed in a single round of replication in the HeLa-CD4-LTR-β-Gal indicator cell line Sx22-1 (Figure 2a), NL4-3 Nef enhanced virion infectivity 4- to 5-fold over a Nef-negative control (Figure 2a). However, changing the original threonine at position 71 to arginine (NL4-3-R) increased infectivity by a factor of three. Strikingly, SF2-T did not augment virion infectivity at all, while SF2-R raised infectivity about 5 times higher than that determined for NL4-3-T. However, given the significant residual infectivity of Nef-negative or T-Nef-encoding viruses, these differences in infectivity are unlikely to account for the absolute dependence on Nef for viral replication in primary cells/DC-T cell cocultures.

Next, we assayed both Nef variants for their ability to downregulate CD4. 293 cells expressing CD4 were transfected with CD8-R/T-Nef chimeras from SF2 and NP003, and steady-state surface expression levels of CD8 (direct internalization of CD8-Nef chimeras) or CD4 (indirect internalization of CD4 by Nef) were determined by FACS and compared with the ED177/178AA mutant defective in both internalization assays [10, 11]. All CD8-Nef isoforms, except the EDAA mutant, showed a similarly reduced surface expression (Figure 2b) as well as similarly reduced CD4 surface levels (Figure 2c). Thus, all isoforms were comparably functional in connecting with the endocytic machinery and downregulating CD4 from the cell surface.

In contrast, the R and T isoforms of Nef differed in their ability to stimulate signaling. Reported effects of this Nef function include the binding and activation of the Nef-

associated PAK kinase [12, 13] and the Nef-dependent increase of Tat transactivation [14]. Binding and phosphorylation of endogenous PAK by R- and T-CD8-Nef chimeras were analyzed through in vitro kinase assays [15]. Whereas both R-Nef isoforms from NP003 and SF2 induced the usually observed strong phosphorylation of p62 (Figure 2d, lanes 2 and 4), the respective T isoforms were impaired (lanes 3 and 5). Similar results were reported previously for the R and T isoforms of NL4-3 Nef [16, 17]. Next, we assayed the activity of suboptimal levels of Tat in the presence of R- and T-Nef (Figure 2e) [14]. Increasing amounts of either isoform of Nef (4 and 8 μg) along with an HIV-LTR-CAT reporter construct and low concentrations of Tat (0.1 μg) were transfected into CV-1 cells. HIV transcription was not altered by the presence of increasing amounts of T-Nef. However, increasing amounts of the R isoform of Nef augmented the Tat effect to about 5-fold over background (Figure 2e). Similar results were obtained with a reporter construct in which the NF-κB site was deleted from the HIV-LTR (data not shown). Taken together, it appeared that the R-Nefs had an increased ability to stimulate cellular signaling as compared to the T-Nefs.

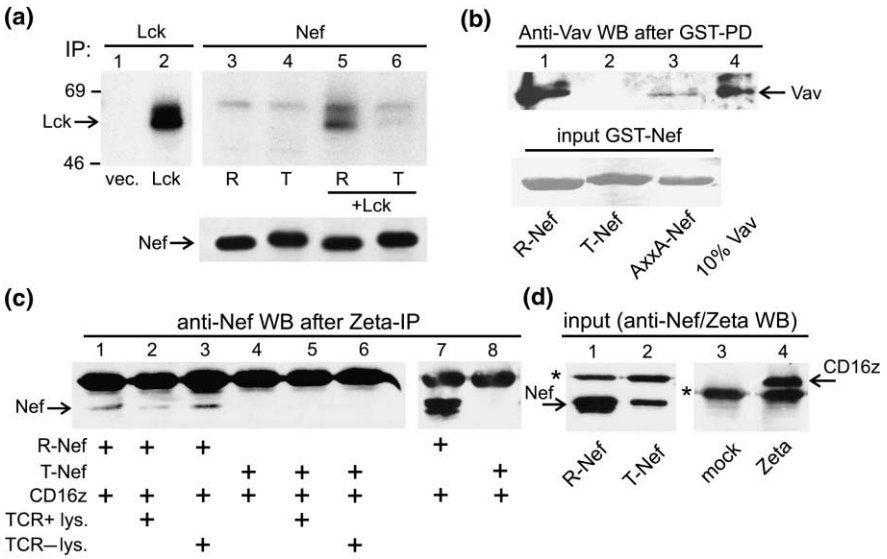
A number of studies reported the interaction of Nef with signaling molecules from the TCR environment, among them Lck, Vav, and the TCR-zeta chain [15, 18–20]. We asked whether both alleles were still capable of binding these signaling intermediates. The interaction of either Nef isoform with Lck was assessed by transient transfections and in vitro kinase assay [15]. Clearly, Lck was coimmunoprecipitated much more efficiently by R-Nef than by T-Nef. (Figure 3a). Next, we tested the interaction of recombinant GST-R/T-Nef with Vav proteins by pull-down assays [19]. The interaction of R-Nef with Vav was readily detected in this system (Figure 3b). Whereas a mutation of the prolines in the PxxP motif maintained some residual binding capacity to Vav, T-Nef was completely negative. Finally, the association of R/T-Nef with TCR-zeta was assayed using recombinant baculoviruses expressing Nef or a CD16-zeta chimera [18]. After coinfection of High-5 cells, CD16-zeta was immunoprecipitated and analyzed for Nef binding by Western blot. R-Nef (Figure 3c, lanes 1–3), but not T-Nef, bound zeta (lanes 4–6) in a specific manner (for details, see figure legend). Less stringent washing conditions increased the binding of R-Nef to zeta considerably (lane 7), but still no association was seen with T-Nef (lane 8). It appeared that the defect of T-Nefs to trigger cellular activation correlated with their reduced ability to interact with the TCR environment.

The differences in electrophoretic mobility between the R- and T-Nefs (Figures 2a,d and 3b) suggested that their functional differences may result from variations in their three-dimensional structure. In fact, by comparing proteolytic degradation and deletion mutants of both isoforms, we found evidence for conformational differences (see Figure S2 in the Supplementary material). The Nef protein adopts a 2-domain structure with a flexible anchor domain (aa 1–56) [21] and a well-folded core domain (aa 57–206) [22–24]. The core domain of NL4-3 Nef contains a flexible N-terminal stretch up to position 80, which includes the PxxP site. This N-terminal region becomes stabilized upon binding to SH3 domains. The comparison of the structures of the T and R isoforms of Nef reveals that, while the threonine points toward the core structure and forms a contact with helix α2 [22, 23], the arginine side chain contributes to the interface with the SH3 domain [24] (Figures 4a,b). A change from the hydrophobic threonine to the hydrophilic arginine destabilizes the anchoring of the PxxP loop to the core structure, likely inducing a conformational change in the SH3 domain binding interface (Figures 4a,b). Detachment of the loop results in a completely accessible PxxP site (residues 58–80) with a higher binding capacity, but does not change the CD4 binding area [25]. Changes in the flexibility of functional loop regions, so called ground-state fluctuations, are known to influence protein interaction [26], e.g., by induced-fit mechanisms. The enlarged accessibility of the PxxP motif, for example, could lead to an increased recognition of SH3 domains (e.g., of Vav). In fact, a completely flexible PxxP site may act as a blueprint for potential proline binding ligands. These conformational differences between R- and T-Nef may explain their divergent functional pattern.

Cultures of immature dendritic cells and T cells, initially established for SIV, impressively revealed the positive effects of Nef on viral replication [4]. Using two naturally occurring, functionally and conformationally distinct isoforms of HIV-1 Nef, we found that cellular signaling through interaction with the TCR signaling machinery, and not CD4 downregulation, is critical for this Nef effect. Our data are in line with the report by Messmer et al. [4], in which SEB activation rescued SIV replication in the absence of Nef. However, which Nef-induced molecular events lead to T cell stimulation? We suggest that Nef initiates signaling via the recruitment of multiple proteins to the plasma membrane and possibly into detergent-insoluble microdomains [27]. With Lck, zeta, and Vav, Nef interacts with key members of proximal TCR signaling [15, 18–20]. Thus, Nef may coaggregate multiple proteins [13], thereby acting in the sense of a signaling adaptor. This recruitment model requires a flexible structure of Nef, possibly provided by the R, and to a lesser extent, by the T conformation.

As shown here and documented in the literature, T variants (e.g., NL-43) may have a reduced capacity to stimulate cellular activation and viral replication [5, 10, 15, 17]. Of note, these effects appeared to be more pronounced

Figure 3

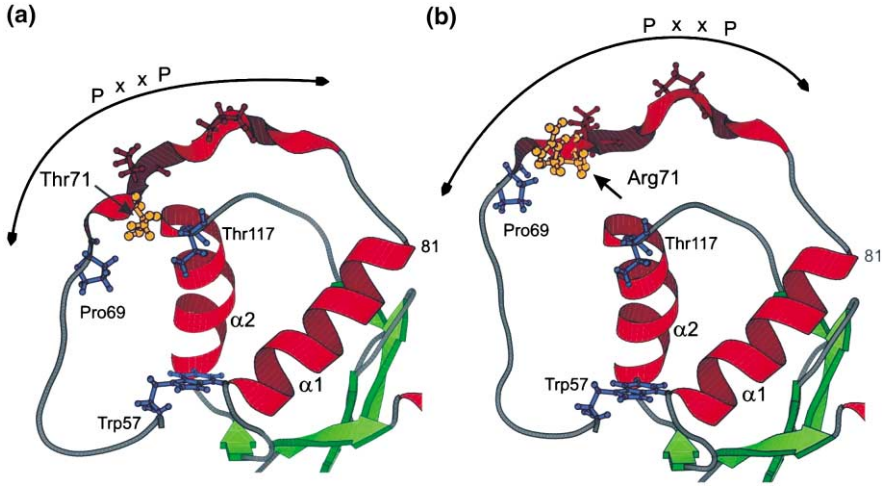


Differential interactions of R- and T-Nef proteins with signaling proteins from the TCR environment. **(a)** An in vitro assay of Lck kinase activity in anti-Lck (lanes 1 and 2) and anti-Nef immunoprecipitates (lanes 3–6) from 293T cells transiently expressing Nef and/or Lck (upper panel). The presence of comparable amounts of Nef protein in these immunoprecipitates was verified by Western blot (bottom panel). **(b)** Anti-Vav Western blot of in vitro binding reactions of GST-Nef and Vav proteins (upper panel). The arrow indicates the position of Vav. Input of comparable amounts of the GST-Nef proteins in the binding reactions was verified by Coomassie staining of the same nitrocellulose filter (bottom panel). **(c)** Anti-Nef (CD8) immunoprecipitation from insect cells infected with the indicated recombinant baculoviruses probed with the anti-zeta antibody. Immunoprecipitations were washed with stringent (500 mM NaCl) (lanes 1–6) or less stringent (100 mM NaCl) buffer (lanes 7 and 8). To compete for zeta binding and show specificity, cellular lysates of Jurkat cells expressing/not expressing the T cell receptor (TCR+/− lys.) were added to the binding reaction (also see [18]). **(d)** Input control Western blot. The asterisks denote the heavy chain, and arrows indicate the positions of Nef and zeta, respectively.

with the Nef protein from SF2 and NP003 than from NL4-3 (Figure 2). This is possibly due to some compensatory mutations in the lab-adapted NL4-3 variant. Since CD4 downregulation was not affected, T-Nefs represent functional proteins; but for what reason are they impaired in promoting viral replication?

For a fraction of an expanding virus population, there may be two reasons not to replicate right away once transmitted by a dendritic cell. First, infected T cells could be used as vehicles that spread the virus in the infected host. In contrast, a replicating virus impairs the migrating capacity of the host T cell and would constitute a recognizable target for the immune defense. Second, T-Nefs may be helpful in establishing a latent reservoir for HIV. Until not activated by strong external stimuli, T alleles may

Figure 4



A model of the different conformations of the R and T isoforms of Nef. **(a)** Part of the NMR structure of the Nef protein from HIV-1_{NL4-3} (T-Nef) starting with residue A56 and including the SH3 binding site (PxxP), helices α1 and α2, and the β-pleated sheet [22]. The PxxP loop from residue 57–80 exhibits several contact points, including the critical T71, to residues in the core domain of Nef. **(b)** A model of the conformation of an R-Nef protein based on the crystal structure of the HIV-1_{NL4-3} T71R Nef mutant [24]. The highly hydrophilic and positively charged arginine at position 71 does not mediate a contact to helix α2 but rather is accessible and interacts with the SH3 domain. This conformational change weakens the contacts of the PxxP loop to the core structure and increases the exposure of the PxxP motif.

exert immune evasion functions (such as MHC downregulation) and keep the virus in latency. In contrast, an increase of viral spread by R-Nefs may be of particular importance at early stages of infection, i.e., efficient transmission of the virus. In conclusion, the high genetic variability of HIV gives Nef a conformational and functional plasticity, perhaps much needed in response to the host immune defense.

Supplementary material

Supplementary material including additional experimental evidence for functional and conformational differences between the R and T isoforms of Nef as well as a detailed Materials and methods section is available at http://images.cellpress.com/supmat/supmatin.htm.

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